Exhibit 8

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Preferential Relative Porphyrin Enrichment in Solar Keratoses upon Topical Application of δ-Aminolevulinic Acid Methylester

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ABSTRACT

Topically applied δ-aminolevulinic acid is used efficiently for the treatment of solar keratoses by photodynamic therapy. Recent animal studies suggest that porphyrin sensitization of epithelial tissue is improved by using esters rather than free δ-aminolevulinic acid. The present study examines porphyrin metabolite formation after topical application of δ-aminolevulinic acid or δ-aminolevulinic acid methylester in human solar keratoses *versus* adjacent normal skin. Levels of total porphyrins, porphyrin metabolites and protein were measured in skin samples excised after 1 and 6 h. Higher levels of porphyrins were observed in solar keratoses than in normal skin with both substances. Maximum porphyrin levels were present in solar keratoses treated with δ-aminolevulinic acid for 6 h. However, the ratio of porphyrins in solar keratoses *versus* adjacent normal skin was higher with δ-aminolevulinic acid methylester. The pattern of porphyrins showed no significant difference between normal and afflicted skin, protoporphyrin being predominant. The results suggest that application of free δ-aminolevulinic acid may be more effective in sensitizing solar keratoses. However, treatment with δ-aminolevulinic acid methylester leads to a preferential enrichment of porphyrins within lesional skin.

INTRODUCTION

Photodynamic therapy (PDT)† with δ-aminolevulinic acid hydrochloride (ALA) is based on the administration of ALA to the diseased skin and subsequent irradiation of the porphyrin-sensitized tissue with red light (1). Tumor necrosis is induced by reactive oxygen species targeting biological membranes as well as by vascular damage leading to tumor cell anoxia (2). Topical ALA-PDT was shown to be highly effective in the treatment of solar keratoses (SK), superficial basal cell carcinoma and superficial squamous cell carcinoma (1,3–6) without serious adverse effects (7).

Accumulation of high intralesional porphyrin levels is essential for effective ALA-PDT. Previously, we showed that irradiation 1–6 h after ALA application was optimal in superficial epithelial skin tumors in relation to adjacent normal skin (8). However, the homogeneity of porphyrin sensitization of neoplastic cutaneous tissues needs to be augmented for improved response rates in ALA-PDT. Higher lipophilicity of ALA esters permits more effective penetration of cutaneous tissue as compared to free ALA. In a recent study, the topical application of ALA esters led to higher porphyrin fluorescence in mouse skin when compared with ALA hydrochloride (9).

Therefore, here we examined the formation of porphyrins after topical application of ALA and ALA methylester (ALA-ME) in SK and adjacent normal skin (NS).

MATERIALS AND METHODS

Materials. Tissues samples of SK and NS adjacent to the lesions (each n = 40) were obtained from patients who underwent surgery. Tissue samples were taken as follows: without substrate application (n = 8), 1 h after application of ALA (n = 8) or ALA-ME (n = 8) and 6 h after application of ALA (n = 8) or ALA-ME (n = 8). One part of the excised tissue was examined histopathologically. Each patient received comprehensive information about the scope of the study.

ALA treatment and photodynamic diagnosis (PDD). Based on pilot studies of topical ALA application (8) and on clinical experience in ALA-PDT (3,4,6), of 20% ALA hydrochloride (Merck, Darmstadt Germany) or the short-chain methyl ester (Sigma Aldrich Chemie, Deisenhofen, Germany) were mixed in an ointment (Neribas®, Schering, Berlin, Germany) and 0.2 g of this mixture was applied to a 1 cm^2 skin area (=40 mg/cm^2) of SK or NS. Treated skin was covered with an occlusive foil (Tegaderm®, 3M Healthcare, Borken, Germany), gauze, aluminum foil and tape to enhance tissue penetration and avoid photobleaching of porphyrins. After 1 or 6 h, the ointment was removed and the treated area was illuminated with Wood's light (Fluotest®, Xenotest, Hanau, Germany; 370–405 nm). Detected fluorescence intensity was expressed semiquantitatively relative to a fluorescence standard. The fluorescent area was marked. Basal values were obtained from untreated controls.

Preparation of skin samples. Only superficial layers of skin (<1 mm) were included in the study because of the limited penetration of topically applied ALA (10–12). Immediately after excision, tissue samples were frozen in liquid nitrogen and stored at −80°C.

Determination of total porphyrin and protein levels. Tissue samples were weighed and cut into small pieces. After homogenization with an Ultraturrax and centrifugation at 3000 U/min for 10 min, porphyrins were isolated with 1.0 *N* perchloric acid/methanol (1/1,

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†*Abbreviations:* ALA, δ-aminolevulinic acid; ALA-ME, δ-aminolevulinic acid methylester; NS, normal skin; PDD, photodynamic diagnosis; PDT, photodynamic therapy; SK, solar keratoses.

Table 1. Fluorescence intensities of tissues treated by δ-aminolevulinic acid or δ-aminolevulinic acid methylester*

	δ-Aminolevulinic acid		δ-Aminolevulinic acid methylester	
	1 h	6 h	1 h	6 h
Normal skin	1.1 ± 0.2	1.9 ± 0.3	0.5 ± 0.2	0.8 ± 0.2
Solar keratoses	1.9 ± 0.2	2.9 ± 0.1	1.4 ± 0.2	1.9 ± 0.2

*Fluorescence intensities of skin treated by δ-aminolevulinic acid or δ-aminolevulinic acid methylester irradiated by Wood's light (370–405 nm, 10 cm distance, 5 mW/cm²). Values are given according to a fluorescence standard: 0 = no fluorescence, 3 = maximum fluorescence (n = 8; mean ± SEM).

vol/vol). In the supernatant, the total porphyrin level was assessed by fluorescence spectroscopy (Perkin Elmer LS-5, Überlingen, Germany); emission was recorded between 520 and 700 nm at an excitation wavelength of 405 nm (Soret band). A protoporphyrin standard was obtained from Porphyrin Products (Logan, UT, USA) (13). Protein levels were determined in the pellet (14).

The relative porphyrin enrichment was expressed by the ratio of porphyrin accumulation in SK *versus* adjacent NS.

Porphyrin metabolites. The supernatant was adjusted with acetic acid to pH 3–4, porphyrins were bound to talcum, esterified and metabolites were identified by HPLC with fluorescence detection (L-7480, Merck Hitachi, Darmstadt Germany) using a porphyrin standard mixture (Porphyrin Products) for quantification (15,16). The following metabolites were analyzed: protoporphyrin, tricarboxylic porphyrin, coproporphyrin, pentacarboxylic porphyrin, hexacarboxylic porphyrin, heptacarboxylic porphyrin and uroporphyrin. The latter four porphyrins are assigned as highly carboxylated porphyrins.

Statistical calculation. Statistical analysis was performed by Student's *t*-test. Data are reported as means ± SEM. Changes were considered statistically significant when the *P* value was <0.05.

RESULTS

Fluorescence intensities of treated tissues

Fluorescence intensity was higher in SK topically treated with ALA for 6 h. Fluorescence was weaker for ALA-ME than for ALA in normal skin (Table 1).

Basal total porphyrin levels

In untreated tissues, the levels of total porphyrins were similarly low: 1.2 ± 0.1 in normal skin and 1.3 ± 0.1 nmol/g protein in SK (Fig. 1 and Table 2).

Total porphyrin levels after topical application of ALA or ALA-ME

Topical application of both compounds led to increased porphyrin levels in SK when compared with NS. Highest levels were detected at 6 h. The ALA treatment induced highest porphyrin values: 36.4 ± 4.0 in SK and 7.2 ± 0.5 nmol/g protein in NS adjacent to the lesions (Fig. 1A). Using ALA-ME as substrate, porphyrin levels in SK were 14.9 ± 2.2 nmol/g protein, which is less than 50% of the amount induced by ALA (Fig. 1B). Furthermore, lowest porphyrin levels were detected in NS treated with ALA-ME (1.7 ± 0.2 nmol/g protein), which is four times less than for ALA.

The relative porphyrin enrichment, *i.e.* the ratio of the level in SK *versus* NS (SK/NS), showed maximum values at 6 h: 5.1 for ALA and 8.7 for ALA-ME (Fig. 2).

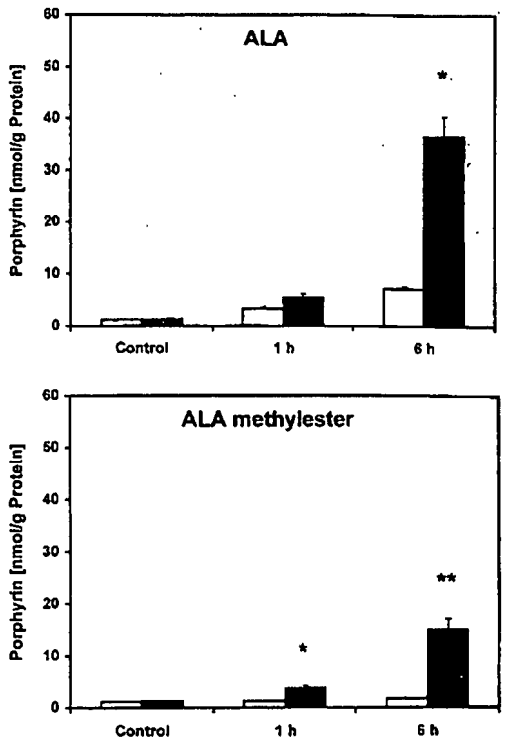

Figure 1. Porphyrin levels in normal skin (NS, open squares) and solar keratoses (SK, filled squares) without and 1 and 6 h after topical application of δ-aminolevulinic acid (ALA) or δ-aminolevulinic acid methylester (ALA-ME). A. Using ALA, in both tissues, maximum porphyrin levels were measured at 6 h. The SK accumulated more porphyrins (36.4 ± 4.0) than NS (7.2 ± 0.5 nmol/g protein; $*P < 0.005$ SK *vs* NS). B. Using ALA-ME, porphyrin accumulation was less than with free ALA (A). In SK increased porphyrin synthesis was detected when compared with NS. Distinct differences already occurred at 1 h: SK 3.7 ± 0.5 and NS 1.3 ± 0.1 ($*P < 0.05$ SK *vs* NS). Maximum porphyrin levels were measured at 6 h (SK: 14.9 ± 2.2 and NS: 1.7 ± 0.2; $**P < 0.01$ SK *vs* NS).

Porphyrin metabolite levels

The basal pattern of porphyrin metabolites was comparable in both untreated tissues with protoporphyrin as the predominant metabolite (89–91%) followed by uroporphyrin and coproporphyrin (Table 2). The pattern of metabolites remained unchanged upon treatment. Protoporphyrin was still the predominant metabolite (82–91%) and accumulated in both tissues over the period of 6 h (Table 2).

DISCUSSION

Porphyrin metabolite formation in human SK and adjacent NS after topical application of ALA-ME *versus* ALA was investigated in the present study. Highest levels of porphyrins are achieved after 6 h in both tissues. Recent biochemical studies on the time course of ALA-induced porphyrin accumulation in epithelial skin tumors showed that maximum intralesional porphyrin levels were measured between 1 and 6 h (8). Therefore, the present study was focused on the application times 1 and 6 h.

Porphyrin accumulation was more intense using ALA than ALA-ME, suggesting that ALA may penetrate the epidermal barrier with subsequent conversion into porphyrins slightly

Table 2. Porphyrin levels and distribution of porphyrin metabolites 6 h after substrate application*

	Substrate	Total porphyrins (nmol/g protein)	Proto-porphyrin (%)	Copro-porphyrin (%)	Pentacarboxylic porphyrin (%)	Hexacar-boxylic porphyrin (%)	Heptacar-boxylic porphyrin (%)	Uroporphyrin (%)
Normal skin	No	1.2 ± 0.1	91 ± 7	3 ± 1	1 ± 1	0 ± 1	0 ± 4	1 ± 3
	ALA	7.2 ± 0.5	90 ± 2	0 ± 1	0 ± 0	0 ± 0	4 ± 1	7 ± 1
	ALA-ME	1.7 ± 0.2	89 ± 2	0 ± 0	0 ± 0	1 ± 0	4 ± 0	6 ± 1
Solar keratoses	No	1.3 ± 0.1	89 ± 7	3 ± 3	0 ± 0	0 ± 1	1 ± 1	7 ± 9
	ALA	36.4 ± 4.0*	90 ± 1	1 ± 1	0 ± 0	2 ± 1	2 ± 0	3 ± 1
	ALA-ME	14.9 ± 2.2†	82 ± 4	8 ± 2	0 ± 1	2 ± 1	5 ± 1	5 ± 1

* Data are given as nmol porphyrin/g protein and percent total porphyrins (n = 8; mean ± SEM; *$P < 0.005$, †$P < 0.01$: solar keratoses *vs* normal skin). Porphyrin patterns showed no significant differences between solar keratoses and normal skin. ALA = δ-aminolevulinic acid; ALA-ME = δ-aminolevulinic acid methylester.

more efficiently than ALA-ME, which requires hydrolysis after penetration. However, using ALA the lesion-adjacent normal skin also accumulated relatively high porphyrin amounts. Thus, topical application of ALA-ME diminishes porphyrin sensitization in normal skin. Regarding the relative enrichment in porphyrin levels SK/NS, ALA-ME seems preferentially to penetrate the damaged skin and abnormal cells of SK (Fig. 2). The low levels of porphyrins in perilesional NS might be primarily due to weak penetration of ALA-ME through intact skin, less formation of porphyrins out of the ester form due to slower deesterification in normal keratinocytes or lower leakage of intralesional formed porphyrins into the perilesional tissue as previously postulated for ALA (8).

The formation of porphyrins after topical application of ALA esters was recently investigated in murine skin (9). All ALA derivatives studied there (methylester, ethylester and propylester) showed higher induction of porphyrin fluorescence than free ALA, particularly after a prolonged application time of 14 h. In contrast, our human *in vivo* data show that NS is less sensitized with porphyrins using ALA-ME when compared with ALA. The use of different species and the lack of neoplastic tissue in the mice might explain the different results. It is known that perilesional NS, as examined in our study, accumulates about three times more porphyrin than NS distant from lesions such as squamous cell carcinomas when treated topically by ALA (8). Thus, it is surprising that the NS localized close to the SK accumulated only low porphyrin levels upon treatment with ALA-ME. Specificity of ALA-ME *versus* ALA may also differ depending on the time of application.

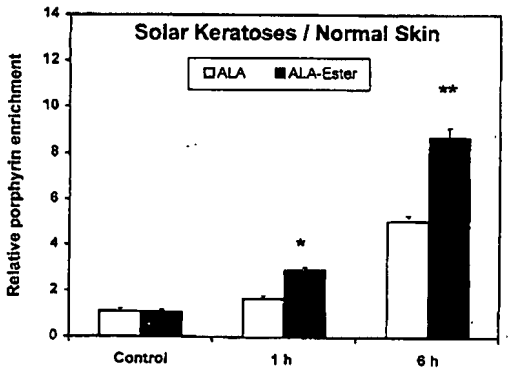

Figure 2. Ratio of total porphyrins in solar keratoses (SK) *versus* normal skin (NS) after topical application of 20% δ-aminolevulinic acid (ALA; open squares) or δ-aminolevulinic acid methylester (ALA-ME; filled squares). The ALA-ME treatment produced significantly higher ratios of porphyrin levels (SK/NS) when compared with ALA treatment: at 1 h the ratio was 2.9 (*$P < 0.05$ ALA-ME *vs* ALA) and at 6 h 8.7 (**$P < 0.01$ SK *vs* NS) was measured.

The porphyrin metabolite pattern shows that any disturbance of the heme-biosynthesis-associated enzymes seems to be not relevant in human SK. The accumulation of higher total porphyrin levels appears to be the major effect responsible for intralesional photosensitization.

The penetration depth of porphyrin fluorescence induced by topical ALA was shown to be inhomogenous (10–12,17–19). Further studies have to show whether ALA esters are superior to free ALA with respect to homogenous porphyrin formation in neoplastic lesions.

In topical ALA-PDT, local photosensitivity commonly lasts up to 48 h (6). The higher lesional selectivity of ALA-ME-induced porphyrins provides for a reduction in cutaneous light sensitivity, particularly in perilesional NS (8). Based on *in vitro* data it might be speculated that long-chain ALA esters are more efficient in PDT than ALA or short-chain ALA esters (20). Optimum efficacy of topical PDT may be expected if irradiation of SK is performed 6 h after treatment with ALA-ME. Application of ALA-ME also optimizes photodynamic diagnosis (PDD), a novel effective diagnostic modality in tumor detection and demarcation (5,6,21,22).

Therefore, the use of ALA-esters in PDT and PDD seems to be a promising modality with highly selective intralesional porphyrin formation.

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